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Review

Direct evidence for cholesterol crystalline domains in biological membranes: role in human pathobiology

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Abstract

This review will discuss the use of small-angle X-ray diffraction approaches to study the organization of lipids in plasma membranes derived from two distinct mammalian cell types: arterial smooth muscle cells and ocular lens fiber cells. These studies indicate that cholesterol at an elevated concentration can self-associate and form immiscible domains in the plasma membrane, a phenomenon that contributes to both physiologic and pathologic cellular processes, depending on tissue source. In plasma membrane samples isolated from atherosclerotic smooth muscle cells, the formation of sterol-rich domains is associated with loss of normal cell function, including ion transport activity and control of cell replication. Analysis of meridional diffraction patterns from intact and reconstituted plasma membrane samples indicates the presence of an immiscible cholesterol domain with a unit cell periodicity of 34 Å, consistent with a cholesterol monohydrate tail-to-tail bilayer, under disease conditions. These cholesterol domains were observed in smooth muscle cells enriched with cholesterol in vitro as well as from cells obtained ex vivo from an animal model of atherosclerosis. By contrast, well-defined cholesterol domains appear to be essential to the normal physiology of fiber cell plasma membranes of the human ocular lens. The organization of cholesterol into separate domains underlies the role of lens fiber cell plasma membranes in maintaining lens transparency. These domains may also interfere with cataractogenic aggregation of soluble lens proteins at the membrane surface. Taken together, these analyses provide examples of both physiologic and pathologic roles that sterol-rich domains may have in mammalian plasma membranes. These findings support a model of the membrane in which cholesterol aggregates into structurally distinct regions that regulate the function of the cell membrane.

Keywords: Cholesterol; Domain; Lipid; Membrane; Atherosclerosis; Lens

1. Background for studies with biologic membrane systems

Free cholesterol is an important structural component of the mammalian cell plasma membrane, where it regulates lipid bilayer dynamics and structure by modulating the packing of phospholipid molecules [1–4]. The cholesterol molecule is oriented in the membrane such that the long axis lies parallel to the phospholipid acyl chains, increasing order in the upper acyl chain region of the membrane while decreasing packing constraints at the terminal methyl groups [2,5]. By restricting the random motion of membrane lipids and the mean cross-sectional area occupied by neighboring phospholipid acyl chains, cholesterol has a pronounced condensing effect on biological membranes [6]. In artificial membrane studies, increasing the amount of cholesterol results in an increase in membrane structural order at temperatures above the phase transition temperature (T_c) and a decrease in structural order at temperatures below the phase transition [1].

1.1. Cholesterol domain in atherosclerotic vascular cell membranes

In certain disease states, including atherosclerosis, elevations in serum cholesterol levels lead to its abnormal

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deposition in vascular cell membranes within the arterial wall. In the mature atherosclerotic plaque, cholesterol has also been observed within the extracellular space in the form of microscopic crystals that can be detected by electron microscopy [7]. These crystals contribute to the mass of the atherosclerotic lesion and trigger inflammatory pathways that result in both necrotic and apoptotic forms of cell death [7]. The mechanism by which cholesterol aggregates and forms crystalline structures in the vessel wall is the subject of intensive investigation.

In animal models of atherosclerosis, it has been demonstrated that the cholesterol content of membranes associated with vascular smooth muscle and macrophage foam cells becomes elevated. At a critical concentration of free cholesterol, self-association of the sterol results in segregation into discrete domains within the plasma membrane [8,9]. These highly organized cholesterol structures, characterized by a unit cell periodicity of 34 Å, may serve as nucleating sites for the formation of extracellular crystals [8,9]. These domains have been previously described in model membrane systems [9–11]. A recent study with cultured mouse peritoneal macrophage foam cells indicates that free cholesterol crystals extend from cellular membranes with various morphologies that include plates, needles and helices [8]. With the use of X-ray diffraction approaches, the early stages of crystal formation could be identified in whole cell and isolated membranes obtained from either diseased tissue ex vivo or cultured cells in vitro [8,9]. Preventing crystal formation is an important goal as cholesterol in this state is practically inert and does not respond well to pharmacologic interventions that promote lesion regression [7,12].

1.2. Cholesterol domains in ocular lens membranes

The presence of cholesterol domains has also been observed in plasma membranes derived from another mammalian tissue source: the human ocular lens. However, it appears that the formation of such structurally distinct cholesterol domains is essential for normal ocular function, including light transparency. The ocular crystalline lens allows for light to be efficiently transmitted through the eye and focused onto the retina through the controlled regulation of its shape. The lens consists of an encapsulated structure consisting almost entirely of a large number of rigid, elongated cells known as lens fibers or fiber cells. These cells are produced by the differentiation of a single layer of epithelial cells located just beneath the anterior surface of the lens and are deposited in successive layers—a process that begins in early embryogenesis and continues throughout life. As new layers are formed, existing fiber cells are displaced toward the center of the lens. Mature fiber cells that are compacted into the center of the lens comprise a region known as the *lens nucleus*; cells peripheral to this region, including new and mitotically active cells of the adult lens, are collectively referred to as the *lens cortex*. To prevent excessive light scattering and compromised lens transparency, fiber cells lose all subcellular organelles during their progressive displacement toward the lens nucleus. Consequently, the plasma membrane becomes essentially the only organelle of the adult lens [13]. A unique biochemical characteristic of the fiber cell plasma membrane is its relatively high level of free cholesterol. The cholesterol to phospholipid mole ratio of the fiber cell membrane ranges from 1 to 2 in the cortex of the lens to as high as 3 to 4 in the lens nucleus [14,15]. This stands in sharp contrast to the levels of cholesterol found in other mammalian plasma membranes, which range between 0.5 and 1.0. It was hypothesized that such high levels of membrane cholesterol content would produce the microscopic appearance of "a mosaic of phospholipid and cholesterol patches" [14]. The fiber cell plasma membrane is also distinct from other biologic membranes in that it contains only trace amounts of polyunsaturated fatty acid [16] and, in the human lens, a phospholipid composition of more than 50% sphingomyelin and sphingomyelin derivatives [17,18].

The unusual lipid composition of fiber cell plasma membrane makes it an intriguing biologic system for conducting structural studies. Moreover, fiber cells can be efficiently removed from the lens and plasma membranes isolated for X-ray diffraction analysis. It was predicted, based on previous studies in model membrane systems, that these biologic membranes consisting of such high cholesterol to phospholipid mole ratios would have discrete cholesterol domains. This hypothesis was tested with the use of small angle X-ray diffraction approaches.

1.3. Membrane structural analysis with small angle X-ray diffraction approaches

The use of X-ray diffraction approaches to examine structural properties of biological membranes has been well established over the last few decades. Reports of X-ray diffraction analyses of lipid membranes were first published at about the same time (1930s) as diffraction patterns from protein crystals. Unlike the field of protein crystallography, however, membrane diffraction remained somewhat esoteric until the 1960s, when this area of biophysical research experienced rapid growth [19]. Small angle X-ray scattering approaches have since been used to study the structure of numerous native and model membrane bilayers.

In order to appreciate the use of X-ray diffraction approaches to analyze membrane structure, it is important to consider the *bilayer hypothesis* or *theory*. According to the bilayer theory, lipids that comprise a membrane are arranged in a bilayer structure as a result of their amphipathic properties. All typical membrane lipids have a polar, hydrophilic headgroup region and a nonpolar, hydrophobic fatty acyl chain region. In order to avoid energetically unfavorable interactions with water, lipids will associate with one another such that their headgroups form two surfaces in contact with the surrounding aqueous environment, with their acyl chains excluded to the region between the two surfaces. The acyl chain region of a bilayer formed

in this manner is called the membrane core, while the hydrophilic surfaces are known as the headgroup layers [20]. If cholesterol is present (which is true of almost all naturally occurring membranes), this molecule is positioned almost entirely within the acyl chain region of the bilayer.

This specific arrangement of membrane lipids is important in that it serves as the basis for the structural continuity of a membrane repeat unit. If membranes are "stacked" into multibilayers, this basic bilayer structure becomes a periodic function that yields coherent scattering in diffraction analyses. Numerous X-ray diffraction experiments have been conducted using membrane multibilayers, including myelin membranes [21,22], disk membranes from the outer segments of retinal rod cells [23,24], erythrocyte ghosts [25], and artificial multilayers derived from the sarcoplasmic reticulum [26,27]. It should be noted that some membrane multibilayers, such as nerve myelin membranes and rod outer segment membranes, occur naturally. Fiber cells also appear to be organized into regular, repeating membrane units in the native ocular lens.

X-ray scattering analysis of membranes that are arranged into multiple layers results in a diffraction pattern consisting of discrete peaks called Bragg reflections. These reflections result from the coherent (constructive) scattering of secondary X-rays produced by atoms comprising a sample. Coherent scattering from membranes follows the same rules as required for the diffraction of crystals: (1) the spacing between the scattering planes must be roughly equal to the wavelength of the incident X-rays; (2) the scattering centers (membrane layers) must be spatially distributed in a highly regular manner; and (3) the repeating membrane units must be oriented so that the diffraction angle (θ) satisfies Bragg's law (see equation below). The application of Bragg's law to the diffraction of membrane multibilayers is illustrated in Fig. 1. In this case, the individual lipid bilayer represents the minimum volume of information that is being repeated in the sample (*unit cell periodicity*). The unit cell periodicity, *d*, is often referred to as the *d*-space, and represents the distance from the center of one water space to the next across the lipid bilayer. Additional concepts related to the diffraction of membranes will be addressed in the following section.

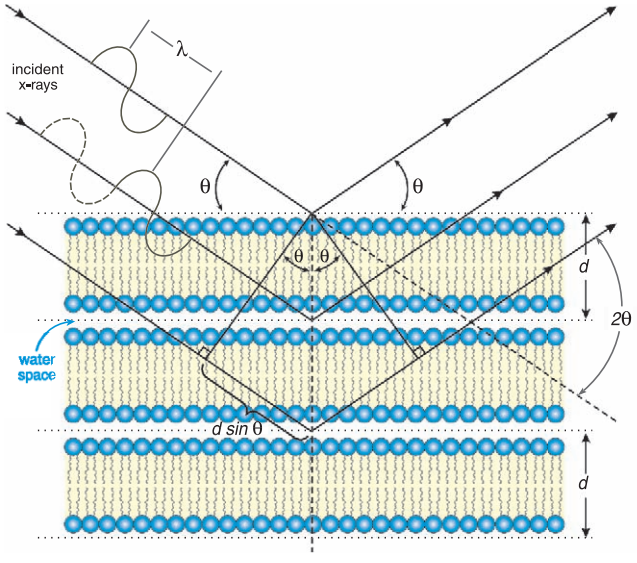

Fig. 1. Schematic representation of membrane bilayers as an X-ray diffraction lattice. The unit cell periodicity, *d*, represents the distance spanning a single bilayer plus half the water space on each side of the bilayer. θ = the diffraction angle.

2. Analysis of biologic membrane systems by small angle X-ray diffraction

2.1. Preparation of vascular smooth muscle cell plasma membranes from models of atherosclerosis

The structure of plasma membranes derived from vascular smooth muscle cells was analyzed by small angle X-ray diffraction approaches. Vascular smooth muscle cells were isolated from a dietary animal model of atherosclerosis. Atherosclerosis was induced in New Zealand white rabbits maintained on a cholesterol-rich diet for up to 13 weeks. The diet was a commercially prepared, calibrated rabbit chow (Buckshire Feeds) supplemented with cholesterol (2%). The chow consisted of 30.1% protein, 5.5% fat, 21.1% fiber and 43.3% carbohydrate (with or without added cholesterol). Control and diet rabbits were housed separately, but in the same room, throughout the feeding period. Control rabbits were fed batch-matched standard chow without added cholesterol.

Freshly dispersed SMC were isolated from the thoracic aorta, as previously described [28]. Following surgical excision, the thoracic aorta was cut open longitudinally and pinned intimal side up to a wax substrate submerged in physiologic salt solution. The intimal surface was scraped with a scalpel to remove endothelial cells and lesions, and the medial smooth muscle layer was peeled free by stripping. The medial layer was minced and incubated for 1–2 h in minimum essential medium (MEM) containing 275 units/ml collagenase, 0.425 units/ml elastase and 0.12% soybean trypsin inhibitor. Dispersed cells were washed in MEM and pelleted at $350 \times g$ for 10 min and used immediately for isolation of membranes.

To confirm cell lineage, an aliquot of cells ($\sim 10^4$ cells) was placed in culture medium and incubated overnight to permit cell attachment, followed by exposure to cell-specific monoclonal antibodies and immunostaining performed with an avidin–biotin system conjugated with horse-radish peroxidase. Confirmation of smooth muscle cell identity was accomplished by immunostaining with the muscle actin-specific HHF-35 monoclonal antibody (Enzo Biochemicals, NY) and macrophage-specific monoclonal antibody RAM-11 (gift from A. Gown). In cells from both normal and atherosclerotic animals, uniform positive staining with the HHF-35 antibody and absence of staining with the RAM-11 antibody were observed, confirming SMC lineage.

Cell dispersions freshly isolated from the aortic medial layer were suspended in cold (4 °C) hypoosmolar sucrose (0.25 M) containing 10 mM Tris (pH 7.4) for 6 min, followed by disruption with a tissue homogenizer (Tekmar #60) using three homogenization cycles. An equal volume of 0.4 M sucrose solution containing 10 mM Tris, pH 7.4, was then added to the disrupted cells. After removal of an aliquot for chemical and enzymatic analysis, the resultant crude homogenate was centrifuged at 268 × *g* for 20 min. The resulting pellet (nuclear/unbroken cells) was resuspended in 0.25 M sucrose, 10 mM Tris, pH 7.4 (as were all subsequent pellets), and the supernatant was centrifuged at 10,950 × *g* for 10 min. The resulting pellet (mitochondrial/lysosomal) was resuspended and the supernatant was centrifuged at 144,000 × *g* for 90 min. The final pellet (microsomal) was resuspended after removal of the post microsomal supernatant (soluble fraction). The identity of the plasma membrane fraction was confirmed with chemical and enzymatic analyses. The ratio of cholesterol to phospholipid under control conditions was 0.4:1.

2.2. Isolation of fiber cell plasma membranes from human ocular lens

The presence of membrane cholesterol domains has also been investigated in the plasma membranes of fiber cells associated with the human ocular lens. The human lens is anatomically separated into two regions: the cortex and nucleus. Human lenses were obtained for these studies from the National Disease Research Interchange (Philadelphia, PA). Lenses were decapsulated with concomitant removal of lens epithelial cells which adhere to the capsule. The decapsulated lenses were placed in a 20-cm^2 culture dish containing 8 ml of 5 mM Tris·HCl (pH 7.4), 5 mM EDTA, 10 mM β-mercaptoethanol (buffer A) and stirred on a rotatory mixer at 100 rpm for 2 h. Under these gentle stirring conditions, the lens cortex separated from the nucleus as clumps of fiber cells. Based on lens weight before and after fractionation, the removed cortex accounted for 48% of total lens volume.

Cortical and nuclear lens regions were separately homogenized in 8-ml buffer A using a glass Dounce homogenizer. Plasma membranes were isolated using the methods previously described [29]. Briefly, the homogenates were centrifuged at 10,000 × *g* for 20 min. The pellets were washed twice with buffer A, extracted twice with 7 M urea in 50 mM Tris·HCl (pH 7.4) and extracted twice with 0.1 mM NaOH. The pellets were then washed once with 5 mM Tris·HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.02% NaN_3 (buffer B) and were resuspended in 1.2 to 1.4 ml of buffer B. Main intrinsic protein of 26 kDa and its 22-kDa degradation product were overwhelmingly the principal proteins shown present by SDS-PAGE in the cortical and nuclear membrane preparations. Total lipids from one half of each membrane suspension were Folch-extracted as previously described and dissolved in chloroform [30]. Aliquots of these membrane lipid extracts were taken for gas-chromatograph quantitation of cholesterol [31] and colorimetric assay of phospholipid [32]. Cholesterol to phospholipid mole ratios for cortical and nuclear membrane samples were calculated to be 2.4 and 3.3, respectively.

2.3. Preparation of oriented intact lens plasma membrane samples for X-ray diffraction

Membranes isolated from either aortic smooth muscle or lens fiber cells were oriented for X-ray diffraction analysis as described previously [33]. Briefly, 200 μg (phospholipid) of plasma membrane samples (in buffer) was loaded into Lucite sedimentation cells. Each sedimentation cell contained an aluminum foil substrate upon which the membrane pellets were collected. The membrane samples were centrifuged in a Sorvall AH-629 swinging bucket ultracentrifuge rotor (Dupont Corp., Wilmington, DE) at 35,000 × *g* for 50 min at 5 °C. Samples were washed three times with diffraction buffer (0.5 mM HEPES, 150 mM NaCl, pH 7.3). After the final washing cycle, the supernatants were removed and the aluminum foil substrates, containing the membrane pellets, were removed from the sedimentation cells and mounted on curved glass supports. The samples were then placed in hermetically sealed brass canisters in which temperature and relative humidity were controlled during X-ray diffraction experiments.

Aliquots of lens plasma membrane total lipid, initially solubilized in chloroform (see above), were added directly to 13 × 100-mm glass test tubes to yield 230 μg of phospholipid. The samples were then dried down under a steady stream of nitrogen gas to the sides and bottom of the test tubes while vortex mixing. Residual solvent was removed under vacuum. A volume of diffraction buffer was added to each test tube to yield a final phospholipid concentration of 0.38 mg/ml. Multilamellar vesicles were formed by vortex mixing the buffer and lens membrane lipids for 3 min at ambient temperature. Volumes yielding the equivalent of 200 μg of phospholipid for each sample were loaded into sedimentation cells. Oriented membrane multibilayers were prepared by centrifugation, as described above for the intact membrane samples.

2.4. Small angle X-ray diffraction analysis

X-ray diffraction analysis of oriented plasma membrane samples was performed as illustrated in Fig. 2. The membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic X-ray beam (CuK_α, $\lambda = 1.54$ Å) produced by a Rigaku Rotaflex RU-200, high-brilliance rotating anode microfocus generator (Rigaku USA, Danvers, MA). The fixed geometry beamline utilized a single Franks mirror providing nickel-filtered radiation ($K_{\alpha 1}$ and $K_{\alpha 2}$ unresolved) at the detection plane. Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Innovative Technologies, Newburyport, MA), the calibration of which was verified using choles-

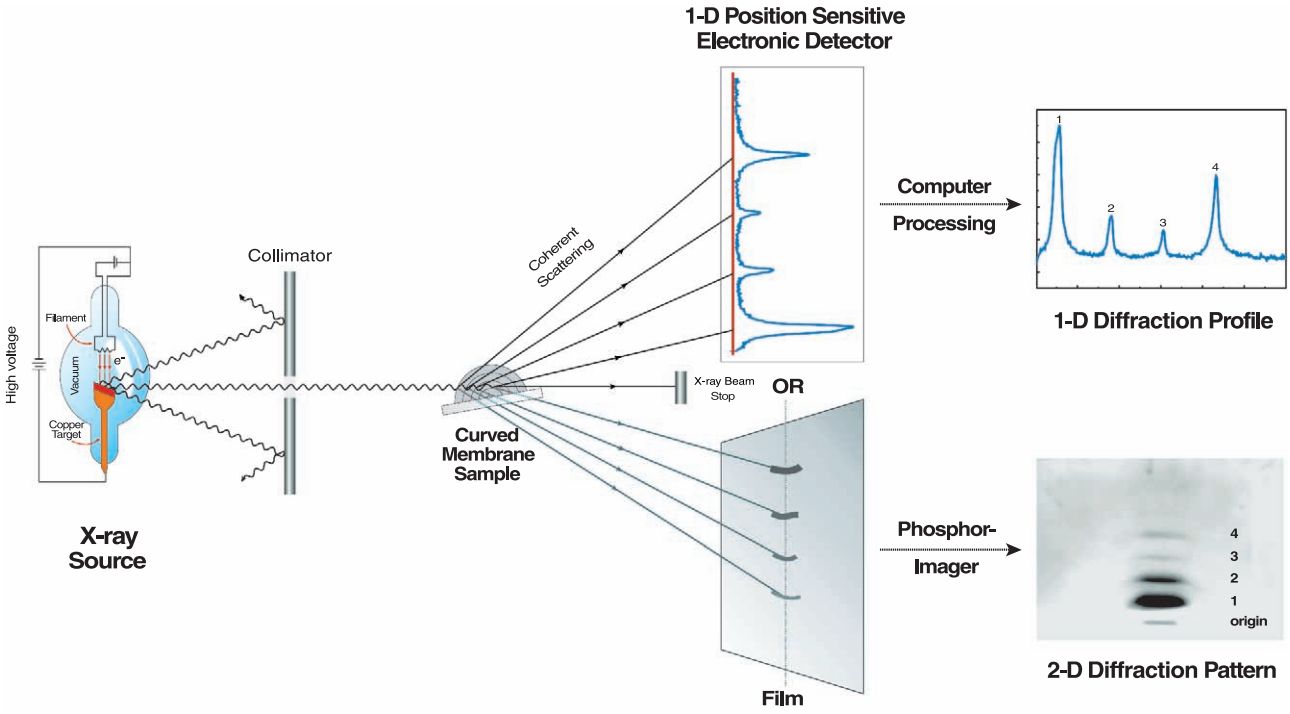

Fig. 2. Schematic representation of the small angle X-ray scattering method. Oriented lens membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic X-ray beam (CuK_a, $\lambda = 1.54$ Å) produced by a Rigaku Rotaflex RU-200, high-brilliance microfocus generator. Coherent scattering data were collected on a one-dimensional, position-sensitive electronic detector. Representative two-dimensional patterns were also collected on Kodak storage phosphor screen film and analyzed using a computerized PhosphorImager system.

terol monohydrate crystals. The sample-to-detector distance used in these experiments was 150 mm. Representative two-dimensional diffraction patterns for each sample were also collected on a two-dimensional PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) at a sample-to-detector distance of 70 mm.

The unit cell periodicity, or *d*-space, of the membrane lipid bilayer is the measured distance from the center of one lipid bilayer to the next, including surface hydration. The *d*-spaces for the membrane multibilayer samples were calculated using Bragg's Law:

$$n\lambda = 2d\sin\theta \quad (1)$$

in which n is the diffraction order number, λ is the wavelength of the X-ray radiation (1.54 Å), d is the membrane lipid bilayer unit cell periodicity, and θ is the Bragg angle equal to one half the angle between the incident beam and scattered beam.

3. Discussion

3.1. Direct evidence for immiscible cholesterol domains in biologic membranes

Earlier views of cell plasma membrane structure were that of a uniform, liquid crystalline organization in which protein and lipid constituents diffuse rapidly in an unrestricted fashion. In recent years, however, new experimental data support a complex, heterogeneous membrane model consisting of microdomains assembled from specific lipid and protein constituents with limited random movement. One type of domain that has been the subject of intensive investigation is the lipid raft, which is enriched in cholesterol and saturated fatty acids, and therefore highly ordered as compared to the surrounding lipid bilayer [34]. Similar to a gel phase membrane, these lipid rafts contain extended acyl chains and melt at high temperatures, but also exhibit rapid lateral mobility as observed for the liquid crystalline phase [35,36]. These lipid rafts consist of specific cellular proteins and mediate a variety of biologic processes, including signal transduction, adhesion and sorting of membrane components. In addition, sterol-rich domains exist within the plasma membrane as structures referred to as caveoli [37].

In this review, we discuss evidence supporting the presence of distinct cholesterol microdomains within the plasma membrane with a reproducible unit cell periodicity of 34 Å. These membrane regions are highly ordered and consist of cholesterol in a tail-to-tail orientation, based on the fact that a single cholesterol monohydrate crystal has a long dimension of 17 Å [38]. These immiscible regions of cholesterol were observed in plasma membrane samples obtained from cells derived from two distinct mammalian tissues: arterial smooth muscle cells and human ocular fiber cells. In membranes obtained from arterial smooth muscle

cells, the presence of cholesterol domains was associated with a pathologic condition (i.e., atherosclerosis) while in the example of the ocular lens the same phenomenon appears to be essential for normal cell function. The identity of sterol-rich regions in these biologic membranes was ascertained by small angle X-ray diffraction approaches (Fig. 3). While the dimension of the cholesterol monohydrate phase remained constant due to its uniform composition and structure, the corresponding dimensions of the sterol-poor lipid phase varied significantly as a function of tissue source and sample conditions (i.e., temperature, relative hydration).

3.2. Effect of sample conditions on structure of sterol-rich versus sterol-poor membrane regions

The differential effect of experimental conditions on the dimensions of the sterol-rich versus -poor regions was observed in fiber cell membranes derived from the human ocular lens. The cholesterol domains were clearly evident in both reconstituted and intact or protein-containing fiber cell plasma membrane preparations (Figs. 4 and 5). For instance, as the relative humidity in the samples was increased, the width of the cholesterol domain remained unchanged at 34 Å. By contrast, the dimensions of the surrounding liquid crystalline phase increased by as much as 30.9 Å (60%) in reconstituted lens plasma membrane. Interestingly, the dimension of the sterol-poor region of the membrane was less affected by experimental conditions in the *intact* fiber cell plasma membranes. Thus, while the presence of protein is not necessary for the formation of immiscible cholesterol domains, it does appear to significantly influence both the size of the cholesterol domains and the dimensions of the surrounding sterol-poor region. In these membrane preparations, the ratio of cholesterol to phospholipid exceeded 2:1 under normal conditions.

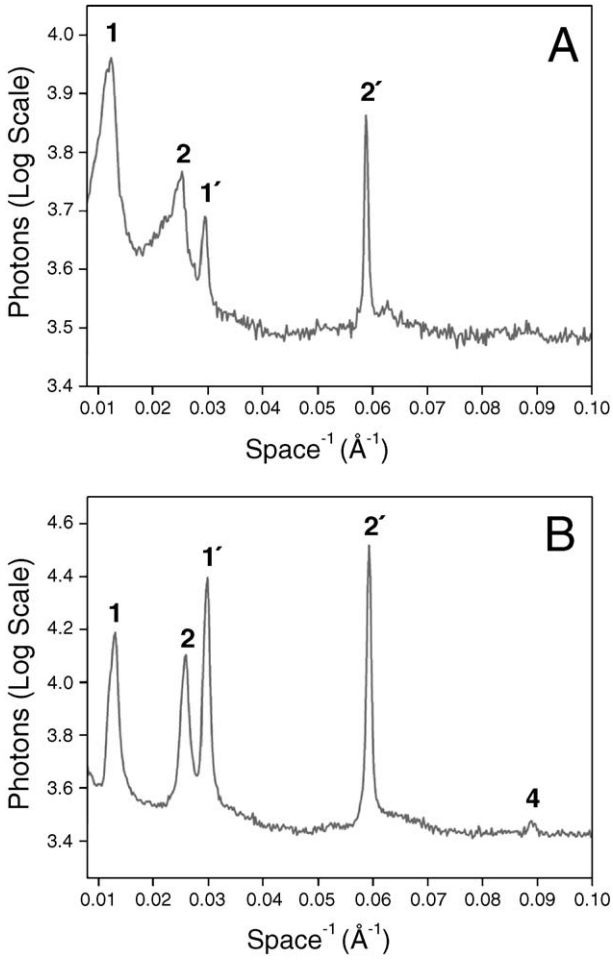

Fig. 4. Representative X-ray diffraction patterns obtained from intact (A) and reconstituted (B) human ocular lens fiber cell plasma membranes. Data were collected on a one-dimensional, position-sensitive electronic detector at 20 °C, 92% RH. Diffraction peaks labeled as 1′ and 2′ correspond to immiscible cholesterol domains; other peaks correspond to the surrounding membrane lipid bilayer.

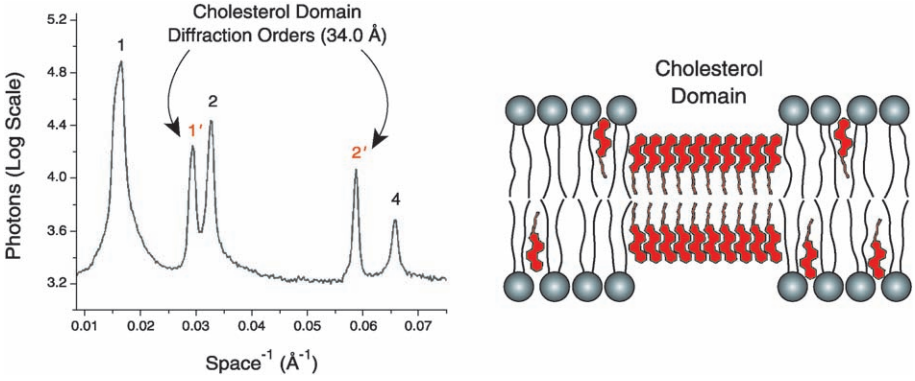

Fig. 3. Identification of membrane cholesterol domains using small angle X-ray diffraction approaches. Cholesterol domains yield characteristic diffraction peaks (1′ and 2′) that correspond to a unit cell periodicity (*d*-space) of 34 Å.

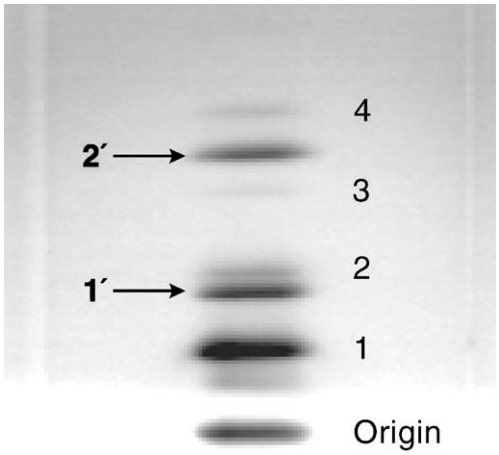

Fig. 5. Two-dimensional diffraction pattern obtained from human ocular lens fiber cell plasma membranes. Meridional diffraction data were collected on X-ray film at 40 °C, 74% RH. Diffraction bands are labeled as for Fig. 4.

3.3. Effects of structurally distinct membrane domains on function in fiber cell plasma membranes and atherosclerotic smooth muscle cells

The functional role for discrete cholesterol regions in ocular lens fiber cell plasma membrane is an intriguing question. The essential activity of the lens fiber cell is to facilitate the efficient transmission of visible light through the eye. By ordering membrane lipid constituents, higher cholesterol levels may provide such transparency. Infrared spectroscopy approaches have demonstrated that the highest membrane cholesterol content is associated with the center or nucleus of the lens where light transmission is greatest [39]. Another role for cholesterol domains may be to interfere with the membrane association of the protein crystallin (especially α-crystallin), an important feature of human and experimental animal cataracts [40]. In fact, cataractogenesis can be accelerated in an animal model by reducing membrane cholesterol content in the lens with specific biosynthesis inhibitors [41]. The results of these membrane structure studies suggest that the coexistence of distinct sterol-rich and -poor regions may interfere with the ability of extrinsic proteins to aggregate at the membrane surface [42].

While the presence of immiscible cholesterol domains is an essential feature of the fiber cell plasma membrane, these same sterol-rich regions are associated with disease in vascular smooth muscle cells during atherogenesis (Fig. 6). The structure of smooth muscle cell plasma membrane was measured in cultured smooth muscle cells and from cells obtained ex vivo from an animal model of dietary atherosclerosis. The effects of cholesterol enrichment on the structure of plasma membranes derived from cells grown in vitro versus those obtained from an intact animal model were remarkably consistent. Following 9 weeks of feeding with a cholesterol-enriched diet, the ratio of cholesterol to phospholipid increased from 0.4:1 to approximately equimolar levels. Under such atherosclerotic-like conditions, prominent cholesterol domains with a unit cell periodicity of 34 Å could be observed in the smooth muscle cell plasma membranes. In both model and biologic membranes, oxidized cholesterol derivatives also formed domains within the membrane lipid bilayer, albeit with different dimensions, as compared to non-oxidized sterol [43]. Unlike the fiber cells, however, cholesterol domains were not observed in normal, non-atherosclerotic membranes.

3.4. Effects of cholesterol enrichment on membrane organization and function

An abnormal accumulation of cholesterol has deleterious effects on membrane function, including the disruption of ion transport mechanisms. Membrane cholesterol enrichment has been demonstrated to affect the activity of various membrane-bound ion channels, including the calcium channel [44] and the Na^+/K^+ ATPase pump [45]. Cholesterol enrichment of the cell membrane also impairs Na^+/K^+ ATPase activity in a variety of cells including erythrocytes [45], endothelial cells [46] and renal cells [47]. In smooth muscle cells derived from an animal model of dietary

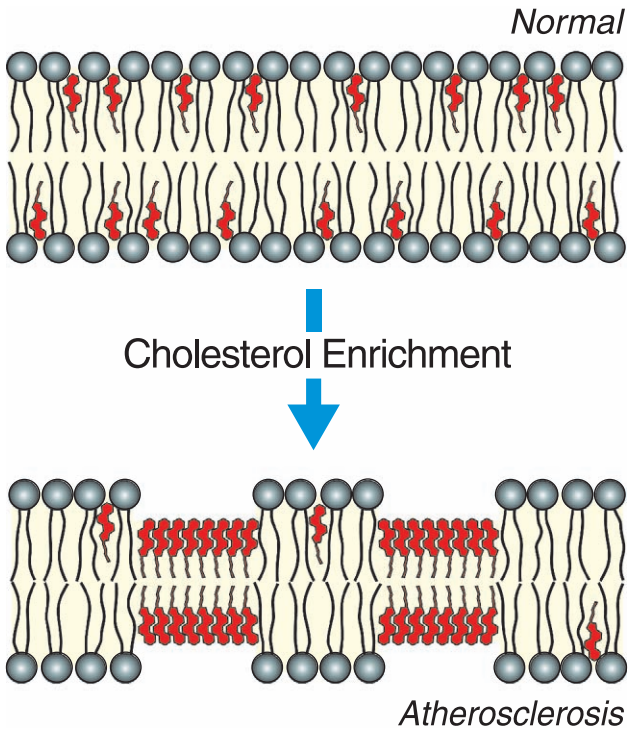

Fig. 6. A schematic model describing the formation of immiscible cholesterol domains in atherosclerotic-like smooth muscle cell plasma membranes following cholesterol enrichment. The cholesterol microdomain has a unit cell periodicity of 34 Å and coexists in the same plane as the surrounding sterol-poor region. Formation of cholesterol domains was observed in vascular cell membranes isolated from animals on a cholesterol-enriched diet and could be reproduced in a cell culture system.

atherosclerosis, calcium transport mechanisms and basal intracellular calcium levels were observed to change as a function of increased membrane cholesterol content [28]. In single channel electrophysiologic recordings of calcium-activated K^+ channels, cholesterol enrichment favored the closed state of the ion channel pore [48]. The effect of cholesterol enrichment on ion channel kinetics correlated directly with changes in structural stress and lateral elastic stress energy [48]. Collectively, these observations provide compelling experimental evidence for the concept that levels of cholesterol are maintained within a certain physiologic range. When the amount of cholesterol exceeds these normal levels, however, there are adverse consequences on membrane function.

Besides adverse cellular consequences associated with excessive cholesterol accumulation, normal cholesterol levels contribute to proper membrane function. Cholesterol is typically associated with separate kinetic domains (or pools) and is thus considered to be distributed nonrandomly within the plasma membrane [2,49–52]. Regulation of the size and physico-chemical properties of these kinetic domains may influence extra- and intracellular cholesterol transport [49,53]. Investigators have proposed that cholesterol domains may modulate the activity of membrane proteins that localize specifically to cholesterol-rich (e.g., nicotinic acetylcholine receptor, human erythrocyte band 3 protein, glycophorin as well as Na^+/K^+-ATPase) or cholesterol-poor (e.g., Ca^{2+} ATPase) domains (see Mukherjee and Chattopadhyay [54] for review). It has also been hypothesized that sterol-rich regions play a crucial role in cellular function that includes signal transduction, cell adhesion, motility and the sorting and trafficking of membrane components [55–58].

In the membrane, cholesterol tends to aggregate into clusters at cholesterol to phospholipid mole ratios in excess of 0.3 [59] and forms separate domains at ratios in excess of 1 (i.e., 50 mol% sterol) [60]. Numerous theoretical and model monolayer and bilayer studies have demonstrated that the systematic addition of cholesterol to biological membranes can eventually yield to lateral phase separation and the formation of membrane restricted sterol domains [9–11,59–63]. In well-defined lipid monolayer systems, the addition of cholesterol produces lateral sterol domains, as characterized by microscopy approaches [61–63]. Membrane cholesterol domains have also been characterized structurally in previous small angle X-ray diffraction studies at high resolution. Shipley's laboratory previously demonstrated that at relative cholesterol levels of 50% (compared to total phospholipid), an immiscible cholesterol monohydrate phase formed with a reproducible unit cell periodicity of 34 Å in coexistence with the liquid crystalline lipid bilayer [10]. This measurement corresponds to a tail-to-tail arrangement of the sterol, as proposed in other model membrane studies using a variety of techniques [64–66]. Based on diffraction studies of myelin membrane, it has been suggested that the membranes also consist of mixed sphingomyelin and cholesterol bilayers [67].

In other studies of nervous tissue, detergent insoluble membrane fractions have been isolated from neuronal cell membranes and shown to have a relatively low sphingomyelin content but a very high cholesterol content [68]. Epand and coworkers showed that the formation of cholesterol-rich domains can be induced by a neuronal protein, namely NAP-22, a myristoylated protein located to the synapse and shown to be a major component of the detergent insoluble, low-density fraction from rat brain. Differential scanning calorimetry experiments demonstrated that NAP-22 changed the shape and enthalpy of phase transitions of phosphatidylcholine and induced the appearance of cholesterol "crystalline" transitions in membranes comprised of either saturated or unsaturated lipids. Using atomic force microscopy, NAP-22 was shown to cause a marked change in the surface morphology of DOPC bilayers containing cholesterol at 0.4 mole fraction. In the absence of protein, the membrane bilayer appeared as a molecular smooth structure of uniform thickness; addition of NAP-22 resulted in the formation of a more convoluted surface, consisting of a raised dendritic domain structure of about 1.5 nm in height [69].

4. Conclusion

This review discussed our use of small-angle X-ray diffraction approaches to evaluate the organization of lipids in plasma membranes derived from distinct mammalian cell types: arterial smooth muscle cells and ocular lens fiber cells. These studies showed that at elevated concentrations, cholesterol can self-associate and form immiscible domains in the plasma membrane, a phenomenon that contributes to both physiologic and pathologic cellular processes. Analysis of meridional diffraction patterns from intact and reconstituted plasma membrane samples indicated the presence of a highly stable, immiscible cholesterol domain with a unit cell periodicity of 34 Å, consistent with a cholesterol monohydrate tail-to-tail bilayer. These cholesterol domains were observed in smooth muscle cells derived from cellular and animal models of atherosclerosis. In fiber cell plasma membrane samples from human ocular lens, by contrast, well-defined cholesterol domains appear to be essential to normal physiology. The organization of cholesterol into separate domains underlies the function of lens fiber cell plasma membranes to maintain lens transparency to visible light while also interfering with cataractogenic aggregation of soluble lens proteins in membrane surface. Taken together, these analyses provide examples of both physiologic and pathologic roles that sterol-rich domains may have in mammalian plasma membranes. Combined with the findings from a various other laboratories, these data support a model of the membrane in which cholesterol aggregates into structurally distinct regions that regulate the function of the cell membrane.

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